Estrogenic activity of biological samples as a biomarker

ESTROGENIC ACTIVITY OF BIOLOGICAL SAMPLES AS A BIOMARKER

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Abbreviations

BPA - bisphenol A

DCC - dextran-coated charcoal

E2 - 17β-estradiol

EDCs - endocrine disrupting chemicals

EEQ - 17β-estradiol equivalent quantity

ER - Estrogen receptor

FSH - Follicle-stimulating hormone

GH - Growth hormone

ISO - International Organization for Standardization

HELN - human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively).

HPLC - high-performance liquid chromatography

LH - Luteinizing hormone

MELN - breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid.

OECD - Organisation for Economic Co-operation and Development

PCBs - polychlorinated biphenyls

PFAA - perfluorinated alkyl acids

POPs - persistent organic pollutants

PP - precocious puberty

TEXB - total estrogenic burden

ABSTRACT

Biological assays can evaluate the cumulative effect of a mixture, considering synergistic/antagonistic interactions and effects of unknown/unconsidered compounds. Therefore, their application could increase in the next years also to analyse biological samples. The aim of this review is to discuss the methodological approach and the application of estrogenic activity assays in human biological samples. 75 research articles were analysed and divided according to whether they used these assays: i) to quantify the level of estrogens and/or as a biomarker of estrogenic status ii) as a biomarker of exposure to endocrine disrupting chemicals (EDCs).

For the first purpose, some authors extracted biological samples while others tested them directly without any treatment. The study of these methodologies outlined that the methodology applied influenced the specificity of analysis. The estrogenic activity biomarker was used to analyse physiological variations of estrogens, pediatric diseases, hormone-dependent diseases and estrogen suppression/enhancement after pharmaceutical treatments.

For the second purpose, some authors extracted samples while others tested them directly, some authors divided endogenous estrogens from xenoestrogens while others tested samples without separation. The analysis of these methodologies outlined some limitations related to the efficiency of extraction and the incorrect separation of some compounds. The studies which applied this EDC biomarker showed that it was correlated with some EDCs, it varied according to the exposure of the population and it allowed the identification of some relationships between EDC exposure and breast cancer, type 1 diabetes and adverse health effects on children.

In conclusion, the estrogenic activity of biological samples can be a useful tool: to quantify low levels of 17β-estradiol, to assess the combined effect of endogenous estrogens and xenoestrogens, to estimate the estrogenic status providing considerable insight into

physiological or pathological conditions, to evaluate EDC presence implementing the existing knowledge about EDC exposure and adverse health effects.

Keywords: estrogenic activity, biological samples, EDC, exposure biomarker, hormone-dependent diseases, adverse health effects.

Contents

1. Introduction

The endocrine system is based on hormones, which are molecules produced by endocrine glands, organs and tissues and released into the blood. Once they reach cells and tissues, hormones can induce several effects through hormone receptors. Hormones are involved in complex signalling pathways, which regulate numerous development stages of human and animal life: foetal development, childhood and puberty. The endocrine system also controls several functions in adulthood such as reproduction, metabolism and thermal regulation, and it interacts with other systems such as nervous and immune systems (Bergman et al. 2013, Demeneix and Slama 2019). Therefore, a correct functioning of the endocrine system is crucial in order to ensure the correct regulation of numerous physiological processes and its dysfunction or perturbation can lead to several adverse health effects such as malformations, metabolic disorders, reduced fertility and cancer (Bergman et al. 2013, Kabir et al. 2015, Pamplona-Silva et al. 2018).

Even if the endocrine system includes several different hormones, the scientific community focused in particular on estrogens because of their peculiar characteristics. Estrogens exert a crucial role in human organisms since they regulate menstrual/estrous reproductive cycles and they are also involved in more complex mechanisms such as the physiology of reproductive

organs and tissues (e.g., breast, ovary and endometrium), lipid metabolism, protein synthesis and diseases (e.g., cancer and neurodegenerative/cardiovascular diseases) (Kiyama and Wada-Kiyama 2015). Moreover, estrogens play a critical role in the physiology and pathology of the immune system, and therefore they can influence the onset and the progression of some autoimmune diseases (Benagiano et al. 2019, Merrheim et al. 2020).

The most important endogenous estrogens are 17β-estradiol (E2), estrone and estriol (Kiyama and Wada-Kiyama 2015) and their actions occur at very low concentrations (Pamplona-Silva et al. 2018).

Due to their key role in the organism, the evaluation of estrogen levels can be helpful to understand and treat a wide range of physiological or clinical conditions in children as well as adults (Rosner et al. 2013). The least invasive methods to estimate estrogen levels are indirect methods which consist in tracking physiologic changes. These methods include monitoring basal body temperature, using tests of urine to detect ovulation, examining vaginal discharge and measuring other body changes, such as uterine length at pelvic sonography to evaluate female pubertal status (Bellem et al. 2011, Paris et al. 2002). However, since they have a low specificity and sensitivity, indirect methods are generally coupled with direct methods such as immunoassays and spectrometry analysis which can quantify hormone levels in biological samples (e.g. saliva, blood, urine) (Bellem et al. 2011). Immunoassays measure hormones using the binding between antigen and antibody, which is amplified using different markers: radioisotope, enzyme, fluorescent or chemiluminescent labels (radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, chemiluminescence immunoassay). Immunoassays are generally considered to be quite specific, but their sensitivity is often insufficient to detect low hormone levels and it seems that they tend to overestimate hormonal levels (Santen et al. 2008). Moreover they are unable to measure different estrogens simultaneously (Bellem et al. 2011) and they do not reflect the hormonal activity in the samples since they only quantify the

concentration of compounds that are structurally recognized by the antibody, thus neglecting the overall activity induced by compounds with the same action mechanism (Widschwendter et al. 2009). Mass spectrometry identifies and quantifies each chemical through its mass-to-charge ratio after ionization (electron spray or electron impact ionization). Before the mass spectrometry analysis, the sample is generally prepared using a separation technique (gas chromatography or liquid chromatography). The tandem mass spectrometry, which is coupled with spectral analysis in multiple rounds, is accepted as the golden standard for hormone assays but it shows some limitations such as the expensive equipment needed and the technical complexity of the analysis, which involves several steps and thus requires a long time (Bellem et al. 2011). Immunoassays and mass spectrometry have been widely used for their specificity since they quantify the concentrations of specific hormones. However, their high specificity may oversimplify the physiological situation. The physiological hormonal activity is mainly based on the effect of a specific hormone, but it can also be induced by other hormones, hormone metabolites and growth factors. Also, it can be altered by exogenous substances. Therefore, in recent years bioassays have been used for hormonal activity quantification by an increasing number of authors. Bioassays are based on biological reactions that depend on the presence or absence of the hormone, but also on the presence of other substances, which can induce the same effect. For example, bioassays for estrogenic activity can detect the total estrogenic effect in human biological samples: they measure the activity of E2 but are also able to detect the activity induced by other estrogens such as estrone and estriol (Paris et al. 2002). In addition to estrogens, a great number of exogenous compounds can exert and modulate the estrogenic activity which can be measured by means of bioassays. These estrogenic compounds are part of a group of substances called endocrine disrupting chemicals (EDCs). The World Health Organization defined EDCs as “exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism,

or its progeny, or (sub) populations" (Damstra et al. 2002). EDCs are a highly heterogeneous group of natural (i.e., steroids and phytoestrogen) and synthesized chemicals (i.e., synthetic chemicals, plastics, plasticizers, pesticides, pharmaceutical agents) (Diamanti-Kandarakis et al. 2009, Kabir et al. 2015) which can interfere with the endocrine system in different ways: first, they can act directly by binding to hormone receptors. In particular, agonist EDCs can imitate hormones, thus producing over or under responses, while antagonist EDCs can block the response. Second, EDCs can indirectly interact with receptors as they can interfere with the synthesis, transport, metabolism and excretion of hormones (Hampl et al. 2016, Kabir et al. 2015). These contaminants are ubiquitous in the environment and human exposure to them occurs in different ways, such as by inhalation (mainly in the working environment), ingestion of contaminated food and/or water and dermal contact through personal care products (Kabir et al. 2015). Since low doses of EDCs could be enough to induce effects - and exposure during specific lifetime periods could induce permanent adverse effects - EDCs may represent an alarming health and environmental problem.

In this context, the assessment of EDC exposure appears crucial in order to monitor populations at higher risk of exposure and to understand the link between exposure and adverse health effects. In biological samples concentrations of EDCs (e.g., bisphenol A (BPA), phthalates, parabens, polychlorinated biphenyls (PCBs), perfluorinated compounds, polybrominated diphenyl ethers) and metabolites of EDCs (e.g., metabolites of pyrethroids, insecticides, pesticides, phthalates) have been used as conventional biomarkers of exposure (Calsolaro et al. 2017, Dziewirska et al. 2018, Hampl et al. 2016, Karwacka et al. 2019). However, since 1995 (Sonnenschein et al. 1995) some authors proposed novel biomarkers of exposure to EDCs, which focus on detecting the biological effect of chemical compounds or metabolites, rather than detecting the presence of the EDC itself. In particular, the estrogenic activity of biological samples, measured with estrogenic activity assays, has been proposed and used as a novel

biomarker of exposure to EDCs. The main advantage of estrogenic activity assays is that they can assess the total effect induced by multiple, exogenous chemicals with estrogenic activity (xenoestrogens). Indeed, the chemical quantification of xenoestrogens provides an estimate of the real exposure. However, the cumulative estrogenic activity of a mixture is not the sum of the individual estrogenic activity of each xenoestrogen, since synergistic/antagonistic interactions must be taken into account. Moreover, the chemical analysis of xenoestrogens only takes into account specific known xenoestrogens and can not quantify the effect of unknown or unevaluated compounds (Bicchi et al. 2009, Escher et al. 2018, Jarošová et al. 2014, Kase et al. 2018, Könemann et al. 2018).

Consequently, the use of estrogenic activity assays in human biological samples may have two main purposes, namely i) the assessment of total estrogenic effect to allow for the management of a wide range of physiological or clinical conditions in children as well as in adults, and ii) the improvement of the assessment of EDC exposure to allow for a better management of this important health and environmental issue.

For the time being the development and application of estrogenic activity assays in human biological samples do not seem to be widespread, compared to other experimental tools. Nevertheless, their use could increase exponentially in the next years due to their versatility and holistic approaches. It is thus much needed to gain additional knowledge of their methodological approaches and applications relevant to them.

The aim of the present review is to discuss the methodological approaches and the applications of estrogenic activity assays in human biological samples. A bibliographic research was performed: 75 research articles were analysed in order to summarize the methods used to treat biological samples and the results obtained.

The examined articles were divided into two categories, according to the use of estrogenic activity: i) to quantify the level of estrogens and/or as a biomarker of estrogenic status, ii) as a biomarker of exposure to EDCs.

To quantify the level of estrogens and/or as a biomarker of estrogenic status, the estrogenic activity assays were applied in 38 of the analysed articles (table 1). Some authors mainly considered estrogenic activity assays as tools for the evaluation of estrogen levels, specifically for the evaluation of E2 levels (table 1, n° ref. 1- 8, 10-13, 18, 29, 32, 38). Other authors instead used these assays for their ability to measure the total estrogenic effect defined as "evaluation of estrogenic bioactivity" (table 1, n° ref. 9, 17). In this review, the application of estrogenic activity assays was intended as a tool to detect the total estrogenic effect, meaning that it was intended as a biomarker of "estrogenic status", except for the articles published by Klein et al. where it was intended as a tool for estrogen quantification. In these 38 studies, serum and plasma were used as biological samples and they were analysed using gene reporter assays (using mammalian and yeast cells) and proliferation assays (E-screen assays).

As biomarkers of exposure to EDCs, estrogenic activity assays were used in 37 of the analysed articles (table 2). In these studies, the biomarker was defined as "assessment of total estrogenic burden (TEXB)" (table 2, n° ref. 3), "total estrogenic xenobiotic burden" (table 2, n° ref. 4), "total effective xenoestrogen burden" (table 2, n° ref. 7, 15, 16, 21, 22, 35) or "xenoestrogenic activity" (table 2, n° ref. 11, 14, 32, 37). In this article the abbreviation "EDC biomarker" will be used. In these 37 studies, serum, adipose tissue, placenta and milk were used as biological samples and they were analysed using ligand-binding assays, gene reporter assays (using mammalian and yeast cells) and proliferation assays (E-screen assays).

2. Assays for estrogenic activity evaluation

Assays for estrogenic activity evaluation have been described in recent review articles (Mueller 2004, Kiyama and Wada-Kiyama 2015, Wagner et al. 2017, Wangmo et al. 2018). For the assessment of the estrogenic activity in biological samples, three kinds of assays have been applied: ligand-binding assays, gene reporter assays and proliferation assays (E-screen assays). In the following sections the characteristics of these assays are briefly described considering their strengths/weaknesses (table 3) and their detection limits (table 4) in the assessment of estrogenic activity in biological samples. The assays validated by the Organisation for Economic Co-operation and Development (OECD) as tools to test potential EDC substances or by the International Organization for Standardization (ISO) as tools to assess water/waste water estrogenic activity are also listed.

2.1. Ligand-binding assays

Ligand-binding assays detect receptor–ligand interactions. These assays quantify the ability of a test chemical or a mixture to compete with E2 in binding to ER, and the result is often expressed as the concentration showing 50% displacement of E2 from receptor. The result can also be expressed as relative binding affinity with respect to E2 (Kiyama and Wada-Kiyama, 2015). Ligand-binding assays were initially performed using radioactive ligands, while, more recently, assays using non-radioactive ligands have been developed. Ligand-binding assays are able to detect the binding to ER of both agonist and antagonist substances without distinguishing between the two effects (Seifert et al. 1999). This characteristic is an advantage. Indeed, while in other estrogenic activity assays agonist and antagonist effects could compensate each other resulting in a null activity, in ligand-binding assays this compensatory effect can not occur. However, the same characteristic can be a disadvantage, since these assays are unable to assess the total estrogenic effect induced by the interaction of agonist and antagonist substances (Mueller 2004).

To assess the estrogenic activity in biological samples, a ligand-binding assay has been applied: the enzyme linked receptor assay (Sapbamrer et al. 2010), a competitive enzyme immunoassay which uses non-radioactive ligands. Moreover, the OECD has validated two ligand-binding assays to screen and test potential EDC substances (OECD 2018), namely the Freyberger-Wilson ER binding assay and the Chemical Evaluation and Research Institute (CERI) ER binding assay (OECD n° 493 2015).

2.2. Gene reporter assays

Gene reporter assays consist of the transfection of a reporter construct into mammalian or yeast cells. ER are transcription factors that induce the transcription of target genes after binding to specific DNA sequences in their promoter; the reporter construct is made of these DNA sequences linked to the gene of a measurable protein (for example the enzyme luciferase) (Sonneveld et al. 2005). Gene reporter assays are able to quantify the total estrogenic effect and are characterized by short incubation periods. However, the results of these assays can be ambiguous when substances with overactivation effects are included in the analysed sample.

Gene reporter assays can be divided into two main categories. Some reporter gene assays are performed on cells that already express ERs, while others are performed on cells that do not express endogenous ERs. In the first category of gene reporter assays, cells are transfected with an estrogen-inducible reporter gene. These cells can be transiently or stably transfected, however, since stably transfected cells remain stable and ready for use, they are generally preferred (Soto et al. 2006, Wangmo et al. 2018). In the second category of gene reporter assays, cells are transfected with both an estrogen-inducible reporter gene and an ER expression construct. Since these cells do not express other ERs, the advantage of these assays is that they allow the evaluation of estrogenic activity in the samples distinguishing between ERα and ERβ estrogenic activity.

Numerous gene reporter assays have been applied for the assessment of estrogenic activity in biological samples. Moreover, two gene reported assays have been validated by the OECD to screen and test potential EDC substances (OECD 2018): the first uses ERα-HeLa-9903 cells and the second uses VM7Luc4E2 cells (OECD n° 455 2016). Both assays use human cells stably transfected with ERα, but the VM7Luc4E2 cells also express a minor amount of endogenous ERβ. As stated by the OECD, VM7Luc4E2 cells were originally designated as the BG1Luc cells, however, in-depth analyses revealed that these cells were not the BG1 cells, but instead a variant of the MCF-7 cells (OECD 2018). Recently, a gene reporter assay using stably transfected human cells has also been described in the ISO 19040-3 (ISO 2018) for the assessment of estrogenic activity in water and waste water. As for gene reported assays based on yeasts, two assays, based on *Saccharomyces cerevisiae* or *Arxula adeninivorans*, have been described in the ISO 19040-1 (2018) and in the ISO 19040-2 (2018) as methods for the assessment of estrogenic activity in water and waste water (Hettwer et al. 2018).

2.3. Proliferation assays (E-screen assays)

E-screen assays are proliferation tests. They measure the proliferative effect induced by estrogens or estrogenic substances on estrogen-responsive cells. Proliferation is determined through different procedures, including DNA staining and metabolic activity quantification. The first E-screen assay was developed by Soto et al. (1997) using human breast cancer cells (MCF-7), whose proliferation is mainly induced by the activation of ERα (Wagner et al. 2017). Later on E-screen assays were also applied using MCF-7 sublines, such as the MCF-7 BUS, in order to obtain a higher proliferative response (Martinez et al. 2016, Villalobos et al. 1995). Many E-screen assays have been applied for the assessment of estrogenic activity in biological samples, while they have not been reported by the OECD nor by the ISO (OECD 2018) to test potential EDC substances or water/waste water estrogenic activity.

3. Estrogenic activity as estrogen quantification and biomarker of estrogenic status: methodologies

The research group of Klein was the first to developed an estrogenic activity assay in biological samples (table 1, n° ref. 1). They developed an estrogenic activity assay as estrogen quantification using yeasts (i.e. a strain of *Saccaromyces cerevisiae)* in which the detection limit was 0.02 pg/mL, in order to overcome the lack of sensitivity of available assays for E2. Klein et al. (1994) treated serum samples performing a liquid-liquid extraction with ether and demonstrated that this assay was highly specific for E2 with low cross-reactivity for estrogen metabolites and other hormones, suggesting that this high specificity could be due to many factors, including the extraction of the samples.

The protocol of Klein et al. was mainly focused on measuring E2 and it was applied in many studies by the same research group (table 1, n°ref. 2-8, 10-13, 18, 22, 29, 32, 38).

Other authors applied extraction to biological samples before the analysis: in the studies of Pedersen et al. (2010) and Chamas et al. (2017) samples were extracted using a liquid-liquid extraction with methyl tert-butyl ether and diethyl ether respectively, while in the study of Kanaya et al. (2015) samples were extracted using a solid-phase extraction.

In 2002, the research group of Paris et al. developed an estrogenic activity assay for the determination of serum estrogenic activity using human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively). In contrast to Klein et al. (1994), Paris et al. (2002) tested serum samples without extraction and without any other sample treatment to keep their conditions closer to those of their physiological status, since their aim was to develop a protocol for the assessment of the total estrogenic activity rather than for E2 quantification. Their protocol, based on HeLa cells, was subsequently applied by several authors (table 1, n° ref. 15, 17, 23, 24, 28) and it was also used by Séronie-Vivien et al. (2004) as a model to develop

another serum estrogenic activity assay based on breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid (MELN).

Other 11 articles reported in this review treated biological samples without any extraction (table 1, n° ref. 16, 19, 20, 25, 26, 27, 30, 31, 34, 35, 37). In almost all protocols, samples were added to culture medium without any treatment. In four studies, however, samples were filtered (pore size 0.22 µm) before being added to culture medium (table 1, n° ref. 20, 25, 30, 32) and an aromatase inhibitor was added with a view to preventing the conversion of testosterone to E2 by aromatase in the cells (table 1, n° ref. 20). Biological samples were tested in one or in different concentrations ranging from 1 to 20% in culture medium.

The analysis of samples without extraction could be influenced by the complexity of serum/plasma samples, therefore some authors proposed different methodological approaches in order to consider the variability of these samples.

The variability caused by serum components was considered by many authors in the construction of the standard curve. In five studies, the standard curve was produced for each sample and consisted of stripped serum from the same patient, to which incremental amounts of E2 were added (table 1, n° ref. 9, 15, 17, 23, 24, 28). Séronie-Vivien et al. (2004) considered this approach as essential when the assay was performed to quantify E2 in serum, because it was useful to remove the interference due to serum compounds different from E2, which modulate the estrogenic activity and differ from patient to patient. However, for the detection of estrogenic activity unrelated to E2 serum concentration, Séronie-Vivien et al. (2004) stated that a single standard curve could be performed using charcoal-stripped serum from a healthy volunteer in order to take into account a "normal overall estrogenic (transcriptional) activity of human serum".

Similarly to Séronie-Vivien et al. (2004), other authors used a single standard curve for all samples (table 1, n° ref. 19, 20, 25, 26, 27, 30, 31). The standard curve was generally

constructed by adding E2 to medium with charcoal-stripped serum/plasma at the same concentration as the sample's one. For example, in the study of Lim et al. (2014a), samples were tested at a concentration of 10% in medium and the standard curve was constructed by adding E2 to 10% charcoal-stripped commercial human serum in medium.

A similar approach was adopted by Sonneveld et al. (2005) in order to test different concentrations of serum samples (0-10%) avoiding the variability caused by serum components. For this purpose, the authors maintained the final serum concentration at 10% by supplementing lower percentages of the tested sera with charcoal-stripped bovine serum.

Data were usually expressed as E2 equivalent quantity (EEQ), which is the total concentration of estrogenic active compounds normalised to the E2. However, Martínez et al. (2016) presented the results as estrogenic activity in comparison with a standard serum pool. In this study, each serum sample was tested with 0.5% serum pool, which was used as a reference standard to normalize the results. Other studies did not specify any peculiar approach to evaluate the results (table 1, n° ref. 35, 37).

Like many hormones, estrogenic activity has a circadian rhythm, with nocturnal and early morning rises (Janfaza et al. 2006, Li et al. 2009). For this reason, some authors pointed out that they collected fasting blood samples during specific hours of the day, especially in the morning (table 1, n° ref. 3, 4, 5, 6, 7, 8, 10, 12, 13, 18, 21, 32, 34, 35, 38).

4. Estrogenic activity as estrogen quantification and biomarker of estrogenic status: applications

Estrogenic activity as estrogen quantification and biomarker of estrogenic status was applied: i) to detect physiological variations of estrogens, ii) to study pediatric diseases, iii) to analyse hormone-dependent diseases in women, iv) to evaluate estrogen suppression or enhancement after pharmaceutical treatments.

4.1. Physiological variations of estrogens

Estrogenic activity assays were used to detect low concentrations of estrogens in children and in post-menopausal women, since the sensitivity of other assays for E2 quantification was too low.

Klein et al. (1994) measured physiological estrogen levels using an estrogenic activity assay in prepubertal children, and found that the estrogen levels in prepubertal girls were higher than in prepubertal boys, suggesting that these hormones may contribute to higher rates of skeletal maturation, earlier puberty and earlier interruption of growth in girls compared to boys. The same results were obtained in the study of Paris et al. (2002) using a different assay for the evaluation of serum estrogenic activity. The role of estrogen levels in skeletal growth was confirmed in another study (Klein et al. 1996): estrogen levels measured in healthy growing boys were low throughout childhood, increased before puberty and rose steadily during adolescence. Moreover, there was a relationship between estrogen levels and testosterone concentrations and between estrogen levels and the time of peak growth velocity.

The physiological variations of estrogens were further evaluated by Janfaza et al. (2006) in 800 healthy children from birth to puberty. In the same study, the estrogen levels were measured every hour for 24 hours in 55 children. Estrogen levels: (i) increased with age and pubertal stage in both genders, (ii) showed a circadian rhythm with a nocturnal rise and (iii) were always higher in girls than in boys.

The estrogenic activity/E2 level of prepubertal children was also evaluated to investigate the relationship between estrogenic activity and adiposity. The first study on this topic was performed by Klein et al. (1998b), whose results revealed that obese and nonobese children in prepubertal or early pubertal stage showed similar circadian rhythms and similar E2 levels. E2 levels were not correlated with fat mass, body mass index, or arm fat, suggesting that E2 levels are not directly correlated with markers of adiposity. Similar results were obtained by Larmore

et al. (2002), who did not find a significant correlation between estrogen levels and body mass index or weight in their analysis of prepubertal and pubertal girls (obese and nonobese). Furthermore, the study of Mesa Valencia et al. (2019) did not show any association between estrogenic activity and markers of adiposity and metabolic and hormonal factors in prepubertal girls, suggesting that estrogenic activity is not influenced by adiposity.

Estrogenic activity assays were also applied to detect low concentrations of estrogens/estrogenic compounds in postmenopausal women. Wang et al. (2005) applied a gene reporter assay using HeLa cells in samples of postmenopausal women, suggesting that such assays could be useful not only to determine the concentration of E2, but also to assess the total estrogenic activity.

Indeed, the authors claimed that the assay was not completely specific for E2 but seemed to measure other biologically active estrogens as well. This hypothesis was supported by a subsequent study (Wang et al. 2013), in which another estrogenic activity assay was applied (E-screen assay). In this study, the serum estrogenic activity of postmenopausal women was strongly associated with serum E2 levels, but it was also positively or negatively associated with other serum molecules and women characteristics. These results suggested that the estrogenic activity is influenced not only by endogenous estrogen levels but also by other factors.

Finally, the estrogenic activity was evaluated in mother-child pairs. The study of Pedersen et al. (2010) evaluated the estrogenic activity of both mother's and child's plasma (cord blood). The results showed that the estrogenic activity of cord blood plasma was higher than the one of mother plasma, and that the two parameters were loosely correlated. The authors also suggested that the high estrogenic activity of cord blood probably reflected the elevated estrogen production in the placenta and was not due to the presence of other estrogenic compounds such as EDCs.

4.2. Estrogenic activity and pediatric diseases

Estrogenic activity assays were applied in order to analyse biological samples collected from children and adolescents with pediatric diseases characterized by hormonal dysregulation: precocious puberty, Turner's syndrome, type 1 diabetes and sex differentiation disorders.

4.2.1. Female precocious puberty

Precocious puberty (PP) is defined as the appearance of secondary sex characteristics in girls aged under 8 years. PP is clinically distinguished between incomplete PP (premature thelarche, premature pubarche, isolated menarche) and true PP (complete or central PP) (Sultan et al. 2018). Estrogenic activity assays have been used on both forms of PP by multiple studies. Three studies focused on incomplete PP and specifically on premature thelarche, which refers to the isolated breast development in girls aged under 8 without the development of any other sexual characteristics. The first study (Klein et al. 1999) performed on young girls (less than 3 years of age) found that E2 levels in girls with premature thelarche were significantly higher than in normal prepubertal girls. In the second study (Paris et al. 2013) information on parental environmental/occupational exposure to EDCs during prenatal/postnatal patient life were collected through surveys, in addition to estrogenic activity evaluation. The estrogenic activity of girls with premature thelarche and whose parents had been exposed to EDCs was significantly higher compared to the one of healthy girls. Moreover, it was significantly higher compared to the estrogenic activity of girls with premature thelarche whose parents had not been exposed to EDCs. This suggested that some patients may be affected by this condition due to prenatal/postnatal EDC exposure. The third study (Pereira et al. 2015) investigated the relationship between estrogen levels and risk of premature thelarche, assessing the estrogen levels of prepubertal girls aged 7, who were monitored during the following years for the onset of thelarche. The results showed that girls with estrogen levels over 5 pg/ml at 7 years had an increased risk of presenting earlier thelarche onset.

E2 levels were evaluated using estrogenic activity assays also in girls affected by true PP. In the study of Larmore et al. (2002), average E2 levels were higher in pubertal girls than in prepubertal girls and in girls with PP. However, only E2 levels of normal pubertal and normal prepubertal girls were statistically different. A high estrogenic activity was also detected in a baby girl (4 months old) affected by precocious puberty (Gaspari et al. 2011b). Since high concentrations of pesticides were detected in the plasma of both the patient and her parents, as well as in the soil of their farm, the authors hypothesized a correlation between her precocious puberty and exposure to pesticides.

4.2.2. Other female diseases

Wilson et al. (2003) compared the serum E2 levels in prepubertal girls affected by Turner's syndrome with healthy prepubertal girls, and found that girls with Turner's syndrome had significantly lower E2 levels. Martínez et al. (2016) compared the serum estrogenic activity of post-menarcheal girls with type 1 diabetes to the estrogenic activity of normal post-menarcheal girls, finding a statistically significant difference in the estrogenic activity of girls with type 1 diabetes compared to the control group. In these two studies, the application of estrogenic activity assay allowed to demonstrate that the lack of normal ovarian function in girls with Turner's syndrome is evident even before puberty, and that type 1 diabetes can also affect estrogen metabolism.

4.2.3. Sex differentiation disorders in males

The study of Paris et al. (2006) found that the serum estrogenic activity of three children with male pseudo-hermaphroditism was higher than in controls. This result, coupled with the mother's exposure to environmental EDCs during pregnancy, suggested that ambiguous genitalia could be related to foetal exposure to EDCs.

Similarly, the study of Gaspari et al. (2011a) found that the serum estrogenic activity of young males with sex differentiation disorder was significantly higher in 11 males who had been

exposed to EDCs during the foetal period compared to 17 cases who had not been exposed and compared to controls. Also, the estrogenic activity of patients who had not been exposed to EDCs was not statistically different compared to controls, suggesting the possible relationship between EDC exposure and sex differentiation disorder of some males.

4.3. Estrogenic activity and hormone-dependent diseases in women

Many studies investigated the relationship between estrogenic activity and breast cancer.

Séronie-Vivien et al. (2004) found that in controls the estrogenic activity was significantly correlated with serum E2 concentration, while in advanced breast cancer patients it was less correlated. In this study, the estrogenic activity was higher in controls than in patients, probably because many pre-menopausal women were included in the control group.

Another study performed a similar comparison using a higher number of subjects (Widschwendter et al. 2009). In contrast with the previous study, the estrogenic activity, measured as estrogen receptor-α and estrogen receptor-β (ERα and ERβ) transcriptional activity, was higher in postmenopausal women with breast cancer compared to postmenopausal controls, although no difference was observed in serum E2 levels between the two groups. Moreover, this study suggested that estrogenic activity assays might predict ER-positive breast cancer at the time of the diagnosis, since women with estrogenic activity (ERα and ERβ) in the highest quintile among controls had 7.57- and 10.14-fold risk of general and ER-positive breast cancer respectively.

Recently, Fourkala et al. (2012) performed a case-control study nested in a cohort study demonstrating that ERα transcriptional activity of serum collected more than two years before diagnosis was independently associated with breast cancer risk, while ERα activity, collected less than two years before diagnosis, and ERβ activity were not. The association between ERα transcriptional activity of serum, collected before diagnosis, and breast cancer risk was also confirmed by the analysis of Asian women in the study of Lim et al. (2014a). Women in the

highest quartile for ERα activity had an odds ratio of 2.39 compared with those in the lowest quartile. As reported also by Fourkala et al. (2012), cases and controls did not differ for ERβ transcriptional activity.

Considering the results of these studies, estrogenic activity assays seem to be a useful tool to assess the risk assessment of breast cancer, since in most of the studies the serum estrogenic transcriptional activity was associated with breast cancer risk.

Fejerman et al. (2016) measured the estrogenic activity of Latin-American women in order to understand whether estrogenic activity may be related to higher breast cancer risk in US-born Latinas compared to foreign-born. Using linear regression models, the authors found a positive association between estrogenic activity and years of US residence (considering foreign-born Latinas only), suggesting that the breast cancer risk observed in Latin-American women might be related to the estrogenic activity.

Starting from this pilot study, Sanchez et al. (2019) assessed the association between estrogenic activity, demographic factors and breast cancer risk factors in American women of different racial/ethnic groups: Non-Latina Black, Non-Latina White and Latina women. Non-Latina Black women showed the highest estrogenic activity, followed by Non-Latina White women, while Latina women showed the lowest estrogenic activity. The multivariable analysis (which included several independent variables) showed that the difference between Non-Latina White and Latina women was statistically significant, as opposed to the difference between Non-Latina Black and Non-Latina White women. The results of these two studies combined suggested that the use of estrogenic activity assays might also provide explanation regarding different breast cancer risk in different racial/ethnic groups living in the US.

The estrogenic activity of serum was also evaluated in relation to another hormone-dependent disease in adults. Lim et al. (2012) considered the possible association between estrogenic activity (measured as ERα transcriptional activity) and hip fracture risk in postmenopausal

551 Asian women. A high serum estrogenic activity, evaluated before hip fracture, was associated
552 with a reduced hip fracture risk. This reduction was still evident after adjustment for other
553 known risk factors of hip fracture were made, including age and body mass index. The results
554 suggested that a high estrogenic activity might prevent this hormone-dependent injury.
555 Finally, the same authors evaluated the influence of serum estrogenic activity (measured as
556 ERα and ERβ transcriptional activity) on lung cancer survival in postmenopausal Asian women
557 (Lim et al. 2014b). Using regression analysis, they found that high ERβ transcriptional activity
558 in sera was associated with a lower probability of lung cancer survival, while on the contrary
559 estrogen levels and ERα transcriptional activity were not associated with the probability of
560 survival. These results suggested that some serum activators of ERβ may lower the probability
561 of lung cancer survival and that the evaluation of ERβ estrogenic activity in sera might serve
562 as a prognostic marker to predict lung cancer survival.

563 **4.4. Estrogen suppression/enhancement after pharmaceutical treatments**

564 **4.4.1. Females**

565 The suppression of E2 levels after pharmaceutical treatments was tested in girls affected by
566 PP. In the study of Klein et al. (1998a), girls affected by PP were treated with different doses
567 of deslorelin for 9 months and serum E2 levels were evaluated using estrogenic activity assay.
568 The results showed that the suppression of E2 was dependent on the dose of deslorelin.
569 However, E2 concentrations were always above the E2 levels of normal prepubertal girls
570 regardless of the dose.
571 Other authors evaluated E2 levels using estrogenic activity assays after pharmaceutical
572 treatments in unhealthy girls. Taboada et al. (2011) assessed pharmacokinetics and
573 pharmacodynamics of the same form of E2 administered orally and transdermally to girls with
574 Turner's syndrome. Girls were treated for two weeks with either a high or a low dose of E2
575 administered orally or transdermally. This preliminary short-term study demonstrated that the

high dose of transdermal administration managed to restore the physiological levels of E2 and estrone, while oral administrations increased estrone concentration compared to both transdermal administration and controls regardless of the dose. High transdermal E2 administration proved to be the most effective way to make the estrogenic activity of the patients the most similar to the estrogenic activity of controls.

This preliminary short-term study was carried forward by the subsequent study of Torres-Santiago et al. (2013). The authors assessed the metabolic effects and body composition changes in girls with Turner's syndrome treated with the same form of E2 given orally or transdermally for a year. E2 doses were titrated in order to achieve E2 levels within the normal range of menstruating adolescents. The two treatments induced similar effects on numerous monitored parameters: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations, body composition (weight, body mass index, percentage of fat mass, fat-free mass, abdominal fat), lipid concentrations and oxidation, resting energy expenditure rates and bone mineral density. However, since the estrogenic activity of serum samples, estrone and estrone sulfate concentrations were higher in girls treated orally with E2 than in girls treated with transdermal E2 administration, the authors concluded that transdermal administration might be more effective than oral administration in inducing a more physiological estrogenic status in girls with Turner's syndrome.

Estrogenic activity assays were also used to test women after pharmaceutical treatments. The first study to focus on women was the one of Klein et al. (1995), in which the E2 levels were measured using a bioassay in women affected by breast cancer and treated with different doses of an aromatase inhibitor for twelve weeks (100 µg/day - 5.0 mg/day of letrozole). The result of the study demonstrated that all doses of the drug induced an equivalent suppression of E2 levels.

The second study on women tested the suppression and recovery of E2 after injection of a potent gonadotropin-releasing hormone receptor agonist (leuprolide acetate) in healthy volunteers (Larmore et al. 2000). Measured with an estrogenic activity assay, E2 levels were significantly suppressed by week 3 and further suppressed by week 4 after one injection of leuprolide acetate. E2 remained below postmenopausal levels for 5-8 weeks after one injection and for 6 weeks after a second injection (4 weeks apart).

Finally, Santen et al. (2002) investigated the estrogenic activity of serum collected from postmenopausal women affected by urogenital atrophy and treated with vaginal E2, in order to determine the lowest dosage needed to reverse signs and symptoms of urogenital atrophy without substantially increasing serum E2 levels. As measured by bioassay, a dose equal to 10 µg of vaginal E2 relieves the symptoms of urogenital atrophy and induces objective vaginal changes without increasing serum E2 levels.

4.4.2. Males

E2 suppression can be induced in boys with severe growth retardation in order to delay puberty and then the epiphyseal fusion, thus maximizing height potential. For this purpose, Mauras et al. (2000) analysed the performance of anastrozole, a nonsteroidal aromatase inhibitor that blocks the conversion of Δ4-androstenedione to estrone and of testosterone to E2. The drug was tested in healthy young boys and the results showed that E2 concentrations were dramatically reduced after anastrozole administration. The drug seemed to be well tolerated and safe, since the treatment did not affect body composition, protein kinetics/substrate oxidation rates, muscle strength, and bone calcium metabolism. Based on these results, the authors stated that anastrozole appears to be suitable to treat boys with growth retardation.

This hypothesis was further supported by the same authors in a subsequent study (Mauras et al. 2004). In this study, growth hormone (GH) deficient boys were treated for 12 months with GH (control group) or with GH and anastrozole in order to suppress estrogen production and

delay epiphyseal fusion. After the treatment, E2 concentrations decreased in boys treated with anastrozole and increased in the control group. Testosterone levels were higher in boys treated with anastrozole compared to control group. Although both treatments did not increase predicted adult height, anastrozole treatment dramatically decreased estrogen concentrations without effects on body composition, plasma lipids, and bone metabolism.

Estrogenic activity assays were also used to assess the effect of the decoction of a Chinese medicinal plant (*Epimedium pubescens*), compared to the effect of a standard estrogenic prodrug (estradiol valerate) generally used to counteract menopausal symptoms (Li et al. 2009). The study was performed on male subjects in order to reduce the interference due to endogenous estrogens, and the estrogenic activity was evaluated in serum using different assays (ERα and ERβ gene reporter assays and E-screen assay). Ingestion of the standard drug induced a significant increase of serum estrogenic activity using all assays, while the plant decoction only induced a small but significant increase of ERα transcriptional activity, suggesting that the administration of this traditional decoction may not be suitable to counteract menopausal symptoms.

5. Estrogenic activity as a EDC biomarker: methodologies

Human biological samples can contain endogenous steroidal estrogens, but also EDCs with estrogenic activity called xenoestrogens, which can be introduced in the human body through inhalation, ingestion of contaminated food/water, and dermal contact. Since the evaluation of estrogenic activity through assays measures the effect induced by all estrogenic compounds, the use of these methods alone is unable to determine whether a phenomenon is induced by endogenous estrogens or xenoestrogens. Therefore, in order to use estrogenic activity assays as a EDC biomarker, in most studies the biological samples were treated in order to remove endogenous estrogens.

650 The studies of Sonnenschein et al. (1995) and Soto et al. (1997) are to our knowledge the first
651 ones that applied estrogenic activity assays as a biomarker. The protocol follows multiple
652 stages: (i) serum sample extraction, (ii) acid cleanup and (iii) separation of xenoestrogens from
653 endogenous estrogens by high-performance liquid chromatography (HPLC) in silica column
654 using the method proposed by Mendina and Sherman (1986). The separation is based on the
655 elution time from the HPLC column. Since xenoestrogens (estrogenic pesticides, PCBs,
656 hydroxylated PCBs, phenolic antioxidants and plasticizers) and endogenous estrogens have
657 different elution times, xenoestrogens can be collected during the first 10 minutes of elution.
658 However, some xenoestrogens, such as phytoestrogens, diethylstilbestrol and mycoestrogens
659 cannot be extracted through this technique, as their retention time is longer than 12 minutes
660 (Soto et al. 1997).
661 The protocol proposed by Sonnenschein et al. (1995) and optimized by Soto et al. (1997) was
662 further refined and modified by Rasmussen et al. (2003) by the introduction of solid-phase
663 extraction and a modified HPLC gradient, with elution of xenoestrogens within 5.5 minutes.
664 Rasmussen et al. (2003), who analysed serum samples from pregnant women, stated that two
665 subfractions of the extract collected during the first 5.5 minutes could contain pregnancy-
666 related hormones (5α- dihydroprogesterone and fatty acid esters of estrone or E2). These
667 subfractions (1.8 – 2.3 min and 3.8 – 4.3 min) were thus removed for the sake of analysis. In a
668 subsequent study, Andersen et al. (2007) collected the xenoestrogens during the first 8 minutes
669 in order to include more hydrophilic pesticides. However, they removed an additional
670 subfraction (6.2–7.1 min) to avoid pregnenolone. The protocol, improved by Rasmussen et al.
671 (2003), was applied in eight other studies (table 2, n° ref. 10, 11, 12, 13, 14, 19, 20, 27).
672 Starting from this protocol, which was focused mainly on the extraction of persistent EDCs,
673 Bjerregaard-Olesen et al. (2015) developed an extraction method for serum samples which was
674 optimized for perfluorinated alkyl acids (PFAA). As the previous one, this method is composed

by solid-phase extraction (water phase), liquid/liquid extraction (tetrahydrofuran/n-hexane) and HPLC fractionation (extract collected between 22.01 and 26.00 min). However, since after the HPLC fractionation estriol and estetrol are still present, this method also contains a weak anion exchange extraction in order to remove these endogenous hormones from the final extract. This protocol optimized for PFAA was subsequently applied in three studies (table 2, n° ref. 33, 36, 37).

Recently, a third protocol with HPLC fractionation was developed in order to analyse human serum samples (Pastor-Barriuso et al. 2016). In this protocol, the HPLC fractionation is preceded by liquid-liquid and solid-phase extractions.

In 1997, the Spanish research group of Rivas and Olea (1997) published another methodology to assess the estrogenic activity as a EDC exposure biomarker using adipose tissue samples instead of serum samples. The authors proposed a protocol in which the adipose tissue was dissolved in hexane and eluted with hexane in a glass column filled with Alumina Merck 90; the eluate obtained was then concentrated and injected in HPLC for separation of xenoestrogens from endogenous estrogens. The HPLC fractionation was performed using the method previously proposed for serum samples (Sonnenschein et al. 1995). Three fractions from HPLC were collected: α-fraction, during the first 11 minutes, containing xenoestrogens; x-fraction from 11 to 13 minutes; β-fraction from 13 to 25 minutes containing endogenous hormones. However, since additives and monomers from plastics (such as bisphenols) are collected alongside endogenous hormones, their effect can not be evaluated analysing the fraction of xenoestrogens.

This HPLC separation protocol was applied by some authors on human adipose tissues (table 2, n° ref. 4, 7, 8, 15, 16, 21, 26, 35), while others applied it on human placentas (table 2, n° ref. 16, 17, 22, 23, 25, 30, 31).

In contrast to previously reported studies, which used extraction and HPLC fractionation, two articles (Natarajan et al. 2002, Sapbamer et al. 2010) separated endogenous estrogens by xenoestrogens with two alternative methods. The former used polyclonal antibodies to immunoprecipitate the endogenous estrogens (E2) and then separate them from the xenoestrogens. In the latter dextran-coated charcoal (DCC) was used to remove all gonadal hormones. The DCC is made from acid washed charcoal powder and dextran and it is generally used to reduce the levels of estrogens in foetal bovine serum, producing the dextran coated-charcoal stripped serum. However, it is important to bear in mind that, as reported by the manufacturer's instructions (Sigma Product Information), although this treatment may reduce the levels of estrogens in the sample it might not completely eliminate estrogens or any other steroids from the serum.

Other studies did not apply any separation technique but extracted the biological sample and tested the whole extract. In the study of Plíšková et al. (2005), half of a crude extract was used for the determination of overall estrogenic activity, while the other half was placed on a sulfuric acid-activated silica column, eluted with n-hexane: diethyl ether mixture, evaporated, and redissolved in DMSO, in order to elute only persistent compounds (including PCBs, polychlorinated dibenzo-p-dioxins and dibenzofurans).

In the studies of Arrebola et al. (2012, 2013), an evaluation without separation was performed in order to assess the combined effect of endogenous estrogens and xenoestrogens, since the estrogenic activity of the whole extract can be considered as a measure of the effect of complex interactions among all estrogenic compounds (i.e. xenoestrogens and endogenous hormones). However, the estrogenic effect of endogenous hormones may be underestimated using this approach. Indeed, as specified by the same research group (Fernández et al. 2007a), this protocol was developed to efficiently extract lipophilic xenoestrogens and, as such, it may not be so effective to extract endogenous hormones. Nonylphenol, octylphenol and BPA, the most

polar xenoestrogens, may also be inefficiently extracted by increasing the under-estimation of the interaction among estrogenic compounds within the sample (Fernández et al. 2007a).

Finally, some studies did not apply any separation techniques and did not extract biological samples but tested them without any treatment as a EDC biomarker (Brouwers et al. 2011, Kanno et al. 2007).

6. Estrogenic activity as a EDC biomarker: applications

The evaluation of estrogenic activity as a EDC biomarker might be a useful tool to understand the relationship between emission, exposure, biological effects and health risks associated with EDCs.

This evaluation can be used for EDC exposure assessment (Andersen et al. 2007) and it can be considered as a biomarker of biological effective dose (Sonnenschein et al. 1995, Soto et al. 1997). However, some authors seem to apply the biomarker also as a biomarker of effect (Bonefeld-Jørgensen 2014), as it can measure the effects induced on the organism by multiple chemicals considering all the possible interactions among them.

6.1. EDC biomarker and exposure to pesticides, polychlorinated biphenyls and perfluorinated alkylacids

Rivas et al. (2001) evaluated the estrogenic activity of adipose tissue extracts containing xenoestrogens (HPLC fractionation technique) collected in 400 women as a EDC biomarker. The concentrations of 16 organochlorine pesticides were also quantified in the extracts, but the concentration of each pesticide was not correlated with the EDC biomarker. The authors claimed that this finding could be due to the combined effect of pesticides and/or to the effect of substances that had not been measured. A subsequent study of Fernández et al. (2004) confirmed that the combined effect of compounds is difficult to predict considering the effect of each one: the authors found that the estrogenic activity of a serum extract (consisting of

749 different extract fractions obtained with HPLC fractionation technique) rarely corresponded to
750 the sum of the estrogenic activity of each fractions.
751 In contrast with the results of Rivas et al. (2001), in the study of Andersen et al. (2007) the
752 estrogenic activity of serum extracts containing xenoestrogens (HPLC fractionation technique)
753 was significantly and positively associated with pesticide exposure evaluated through
754 interviews both with pregnant and non-pregnant women working in Danish greenhouses. The
755 results of this study also demonstrated that the EDC biomarker is representative of recent
756 exposures. Indeed, among pregnant women, the positive association was only significant for
757 those who had been working one week before the sampling, while no association was observed
758 for women working in the previous period.
759 A negative association between PCB exposure and estrogenic activity was observed by
760 Plíšková et al. (2005) who studied men living in a PCB polluted area and in a control
761 background area (different districts in eastern Slovakia). Serum extracts containing persistent
762 compounds (extraction technique optimized for persistent compounds) from people living in
763 the background area showed higher estrogenic activity, while extracts from the polluted area
764 showed an antiestrogenic activity. The association between PCB exposure and antiestrogenic
765 activity was also confirmed by the comparison between the total estrogenic activity and PCB
766 concentrations, since the estrogenic activity of the extracts containing endogenous estrogens
767 and persistent compounds (crude extracts) was lower in the samples with high PCB levels.
768 Since the levels of E2 decreased in the samples with high PCB levels, the authors stated that
769 exposure to high PCB levels might also affect concentration of E2 in blood, causing the
770 decrease of estrogenic activity and an overall antiestrogenic effect.
771 Kanno et al. (2007) studied the estrogenic activity of serum samples (without treatment)
772 collected from patients who underwent hemodialysis and peritoneal dialysis compared to a
773 control group. The patients' sera had higher levels of BPA compared to controls and they

induced a higher estrogenic activity compared to the activity expected for the measured BPA levels. The authors discussed the results, claiming that the high level of BPA was probably due to the release of BPA from the dialyzers to patient's serum and that the unexpectedly high estrogenic activity could be due to the release of other EDCs, such as phthalate diesters in patient's serum.

Brouwers et al. (2011) studied men with different EDC exposure levels and found that occupational exposure to pesticides, disinfectants and exhaust fumes were associated with increased plasma estrogenic activity (without treatment). Instead, body mass index, use of personal care products and proximity to city centre were not associated with the EDC biomarker.

However, the results of Kanno et al. (2007) and Brouwers et al. (2011) could have been influenced by a possible interference of xenobiotics with endogenous estrogen levels, since the authors used the estrogenic activity in total plasma/serum as a EDC biomarker without dividing endogenous estrogens from xenoestrogens.

Finally, Bjerregaard-Olesen et al. (2016) studied the relationship between exposure to PFAAs and estrogenic activity of serum extracts containing PFAAs (HPLC fractionation technique) in pregnant women. Their study found positive linear associations between the estrogenic activity and the PFAA serum levels but also inverse linear associations between extracts with an antiestrogenic activity (when tested with E2) and PFAA serum levels.

6.2. EDC exposed populations

The EDC biomarker was used to study Nordic populations, which are considered to be highly exposed to persistent organic pollutants (POPs) and EDCs, since numerous POPs (such as PCBs, dioxins and organochlorine pesticides) are also classified as EDCs. Due to their resistance to environmental degradation, POPs are transported to the Arctic by the atmosphere and ocean currents; here, due to their chemical characteristics, they are bioaccumulated in the

adipose tissue of animals and then of humans through their diet. Nordic populations are particularly exposed to POPs since their diet consists of a high intake of fish and marine animals (seals, whales, polar bears, seabirds) and includes the consumption of tissues and organs that contain animal fats where POPs are highly accumulated (liver, blubber, skin) (Bonefeld-Jørgensen et al. 2010).

Rasmussen et al. (2003) studied the estrogenic activity of serum extracts containing xenoestrogens (HPLC fractionation technique) collected from three groups of women: 30 pregnant Danish women (slightly exposed), 60 non-pregnant Danish women (slightly exposed) and 211 pregnant Faroese women (highly exposed through diet), and found that the EDC biomarker was higher in Faroese women compared to Danish women.

Other authors studied the Inuit Greenlandic populations as exposed populations, and compared the Inuit men with European men from Sweden, Poland (Warsaw) and Ukraine (Kharkiv). The results of the comparison showed that Inuit serum extracts containing xenoestrogens (HPLC fractionation technique) induced no estrogenic activity but elicited an antagonistic effect when the extracts were tested in combination with E2 (71% of samples) (Bonefeld-Jørgensen et al. 2006). Contrarily, some European extracts induced estrogenic activity and only a few extracts elicited an antagonistic effect when tested with E2 (7-30% of samples). The EDC biomarker was not strongly associated with serum concentration of POPs (Bonefeld-Jørgensen et al. 2006) and no consistent association was found between the EDC biomarker and the adult semen quality assessed as sperm concentration, motility and morphology (Toft et al. 2007). In addition to lower estrogenic activity, Inuits were found to have lower sperm DNA damage, while in Europeans the estrogenic activity was positively correlated with DNA damage, suggesting that altered estrogenic activity together with genetic and/or nutrient factors may protect Inuits' sperm DNA from damage (Long et al. 2007). Different correlations between sperm DNA

damage and the EDC biomarker were further showed when comparing Inuits and Europeans using another assay (sperm chromatine structure assay) (Krüger et al. 2008b).

The EDC biomarker was also used to study differences among Inuit populations across Greenland. The study of Krüger et al. (2008a) measured the estrogenic activity of serum extracts (HPLC fractionation) in men and women from different Greenlandic districts (Nuuk, Sisimiut and Qaanaaq) to evaluate associations between the EDC biomarker, POPs concentrations (14 PCBs and 10 pesticides), and lifestyle characteristics. The EDC biomarker showed different levels depending on districts and genders. In accordance with previous studies, male and female serum extracts generally induced a decrease in estrogenic activity and showed an antagonistic effect when the extracts were tested in combination with E2. Overall, few correlations were observed between the EDC biomarker and concentrations of each PCB and pesticide. A similar experimental study was performed in 2012 by the same authors (Krüger et al. 2012) in other Greenlandic districts (Ittoqqortoormiit, Narsaq and Qeqertarsuaq). The levels of the EDC biomarker were different depending on the district. The Ittoqqortoormiit serum extracts contained high levels of POPs and elicited an antagonistic effect similar to the extracts tested in the previous study (Nuuk, Sisimiut and Qaanaaq). On the contrary, a higher agonistic effect was observed in Qeqertarsuaq and Narsaq extracts. As summarized by two reviews (Bonefeld-Jørgensen 2010, Bonefeld-Jørgensen et al. 2014), the results of studies on Greenlandic Inuit suggested that the EDC biomarker is negatively correlated with POPs and it can be used as a biomarker in order to detect POP exposure.

6.3. EDC exposure and adult adverse health effects

The EDC biomarker was also applied to study the association with type 2 diabetes and breast cancer.

Arrebola et al. (2013) applied the estrogenic activity measured in adipose tissues (extraction technique) of adults from Southern Spain as pesticide and PCB exposure biomarker, in order

to evaluate the possible role of these substances for the onset of type 2 diabetes. In the study, the concentrations of pesticides and PCBs in adipose tissue and serum were associated with type 2 diabetes, while the EDC biomarker was not associated with the disease and did not influence the effect of the substances on the disease, indicating that estrogenic activity may not be a critical factor for the onset of diabetes.

Among the studies on breast cancer, Ibarluzea et al. (2004) evaluated the estrogenic activity of two types of adipose tissue extracts (containing xenoestrogens and containing endogenous estrogens- HPLC fractionation technique) and the concentration of 16 organochlorine pesticides in the adipose tissue in a case-control study, comparing women who had just been diagnosed with cancer and control women. No significant differences were observed in concentrations of pesticides and in the estrogenic activity of extracts containing xenoestrogens between the two groups. However, in women with a body mass index below the median (especially for the postmenopausal group), the high estrogenic activity of extracts containing xenoestrogens was associated with increased risk of breast cancer. The association was detected only by considering the activity of extracts containing xenoestrogens, while no association was found between breast cancer risk and the estrogenic activity of extracts containing endogenous estrogens (Fernández et al. 2007b). This first evidence of a significant association between the EDC biomarker and the risk of breast cancer was further investigated considering potential confounders and covariates (Fernández et al. 2007a). In patients, the estrogenic activity of adipose tissue extracts containing xenoestrogens (EDC biomarker) (HPLC fractionation technique) was associated with age, family history of breast cancer, lactation experience and smoking, while in controls it was only associated with age. Moreover, in patients the estrogenic activity of adipose tissue extracts containing endogenous estrogens (HPLC fractionation technique) was associated with age, educational level, age at menarche, menopausal status, marital status, lactation experience and smoking, while in controls it was

only associated with menopausal status. Therefore, the analysis of confounders and covariates showed that the EDC biomarker might also be sensitive to environmental, dietary, lifestyle, genetic and reproductive factors.

A similar case-control study (Pastor-Barriuso et al. 2016) investigated the relationship between the EDC biomarker and breast cancer risk using serum instead of adipose tissue samples. No significant difference was observed in concentrations of each organohalogenated compound (PCBs, hexachlorobenzene, p,p'-dichlorodiphenyldichloroethylene) between women with breast cancer and controls. In contrast with the previous study, the estrogenic activity of both types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC fractionation technique) was higher in cases than in controls and the estrogenic activity measured in serum extracts was not associated with potential confounders (e.g. age, body mass index) except for geographical region. Nevertheless, in accordance with the previous study, this study highlighted the importance of the EDC biomarker since it demonstrated a strong positive association between the estrogenic activity of extracts containing xenoestrogens and the risk of breast cancer. Moreover, it revealed an association with a sigmoidal trend between estrogenic activity of extracts containing endogenous estrogens and breast cancer risk.

A perspective study was conducted by the same research group in order to evaluate the effect of breast cancer treatment (chemotherapy and/or radiotherapy) and the influence of cancer progression on the EDC biomarker (Fernández et al. 2017). The authors assessed the estrogenic activity of adipose tissue in breast cancer patients at the diagnosis and during four other consecutive periods (<6, 6-12, 12-18, >18 months) and found that estrogenic activity of both types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC fractionation technique) increased during the treatment with a maximum peak reached at 6-12 months, suggesting that cancer treatment might influence the levels of the EDC biomarker.

These studies demonstrated the association of the EDC biomarker with the risk and progression of breast cancer in Spanish women. Nevertheless, the same results were not obtained in a similar study performed on serum samples of Inuit women (Wielsøe et al. 2018). Indeed, in this case-control study, the authors evaluated the estrogenic activity of two types of serum extracts containing lipophilic xenoestrogens and containing PFAAs (HPLC fractionation technique); the results showed that the estrogenic activity of both extracts was not associated with breast cancer risk. This unexpected result can be due to the different assays used for the assessment of estrogenic activity. Indeed, this study applied a gene reporter assay, while the previous studies evaluated the estrogenic activity through the E-screen assay which is a proliferation assay. Moreover, other differences might be responsible for the discrepancy among the results, namely different characteristics among the studied populations, extraction methods and exposure levels.

6.4. Mother EDC exposure and child adverse health effects

Since exposure to EDCs during pregnancy could represent a risk for children, some authors applied the EDC biomarker in order to detect a possible association between the estrogenic activity of placenta/serum extracts collected in mothers and adverse health effects in their children.

To our knowledge, Fernández et al. (2007c) is the first study in which the EDC biomarker was applied for this purpose. The aim of this study was to investigate the mother-child exposure to organochlorine chemicals and its association with the risk of male urogenital malformations. The estrogenic activity was evaluated on placenta samples collected from mothers whose children were affected by cryptorchidism and/or hypospadias (n=46) and from controls. The estrogenic activity of two types of extracts (containing endogenous estrogens and containing xenoestrogens- HPLC fractionation technique), was not associated with the concentration of 16 organochlorine pesticides measured in the extracts. However, the estrogenic activity of

extracts containing xenoestrogens (EDC biomarker) showed a weak association with the risk of malformation, which was stronger when adjusted for maternal age and birth weight. The results of the study suggested that the EDC biomarker may be a risk factor for cryptorchidism (Fernández et al. 2007b).

Other studies evaluated the effects on children of exposure to xenoestrogens during pregnancy. In the study of Vilahur et al. (2013), the estrogenic activity of placenta extracts containing xenoestrogens (HPLC fractionation technique) was positively associated with increased birth weight and with a decrease in the risk of a rapid growth only in boys, although no association was found between the EDC biomarker and body mass index measured at 14 months of age. These results suggested that exposure to xenoestrogens during pregnancy may affect male child health in particular. A sex difference was also observed in two subsequent studies of the same authors (Vilahur et al. 2014a, Vilahur et al. 2014b). The first study showed an association between increasing levels of estrogenic activity in extracts containing xenoestrogens (HPLC fractionation technique) and lower AluYb8 DNA methylation, both measured in placentas of male children, while no significant effect was detected analysing placentas of female children. In the second study (Vilahur et al. 2014b), a lower average on motor development tests at 1-2 years of age was observed in boys with mothers having high estrogenic activity of placentas (extracts containing xenoestrogens- HPLC fractionation technique), while no associations were observed in girls. The association was not found in children at 4-5 years and neither in the analysis of mental and cognitive tests in children at 1-2 years and 4-5 years.

Recently, Bjerregaard-Olesen et al. (2019) investigated the associations between exposure to xenoestrogens during pregnancy (evaluated with the EDC biomarker) and child parameters (i.e. birth weight, length and head circumference). Differently from the other studies, in this study the biomarker was evaluated not in placenta extracts but in serum extracts and an extraction optimized for PFAAs was used (HPLC fractionation technique and PFAA extraction). The

results showed that a higher estrogenic activity of serum extracts containing PFAAs was associated with lower birth weight and length of children, suggesting that PFAA exposure during pregnancy may affect child growth.

Finally, since EDC exposure during the first life period of children can occur through milk, Sapbamrer et al. (2010) studied the relationship between maternal estrogenic activity of serum and activity of breast milk (both treated in order to remove endogenous estrogens-DCC technique) and the correlation between estrogenic activity and lipid levels. The results showed a correlation between serum estrogenic activity and serum lipid levels, while no correlation was found in milk. Moreover, the study demonstrated that the estrogenic activity in breast milk was 8–13.5 times higher than in serum and that the two EDC biomarkers were not significantly correlated, suggesting that child exposure through milk can not be precisely estimated analysing maternal serum.

7. Conclusion

Estrogenic activity assays in human biological samples were applied for two main purposes:

i) as estrogen quantification and/or biomarker of estrogenic status;

ii) as a biomarker of exposure to EDCs.

Different methodologies were applied for the first purpose: some authors extracted biological samples, while others tested them directly without any treatment. Several methodologies were applied in order to obtain different levels of specificity: highly specific protocols with extraction allowed the quantification of low concentrations of E2, while less specific protocols without extraction, allowed the quantification of the total estrogenic activity.

Estrogenic activity as estrogen quantification and biomarker of estrogenic status was useful to detect low concentrations of estrogens/estrogenic compounds and allowed the detection of physiological variations of these compounds in prepubertal children and postmenopausal

women. This biomarker was also useful to study pediatric diseases characterized by hormonal dysregulation and women hormone-dependent diseases. Overall, in many studies a high estrogenic activity was associated with PP in females and sex differentiation disorders in males and in some studies the use of this biomarker allowed the identification of EDC exposure as a possible risk factor for these two diseases. Moreover, in girls the biomarker was also associated with Turner syndrome (one study) and type 1 diabetes (one study), while in women a high estrogenic activity was associated with higher breast cancer risk (three studies), lower hip fracture risk (one study) and lower lung cancer survival (one study). These relationships should be evaluated with caution since they were only demonstrated in a limited number of studies and through the analysis of small groups of subjects. Furthermore, the results may have been influenced by the type of estrogenic activity assay applied: some correlations were statistically significant mainly using a type of estrogenic activity assay (e.g. ERα transcriptional activity was correlated with breast cancer in all studies, while ERβ was correlated only in one out of three studies; ERβ transcriptional activity was correlated with lower probability of lung cancer survival, while ERα transcriptional activity was not). This biomarker was also important to monitor estrogen suppression/enhancement in females and males treated with different drugs: deslorelin, oral E2, transdermal E2, vaginal E2, letrozole, leuprolide acetate, anastrozole, decoction of a Chinese medicinal plant.

For the second purpose, in order to use estrogenic activity assays as a EDC biomarker different methodologies were applied on biological samples: some authors extracted biological samples, while others tested them directly without any treatment; some authors divided endogenous estrogens from xenoestrogens, while others tested samples without separation. The analysis of these methodologies outlined some limitations. Regardless of the technique used for extraction (solid phase extraction, liquid/liquid extraction or both) and separation (HPLC, immunoprecipitation, DCC), the extraction collects different compounds with different

efficiencies depending on the protocol. Thus it may underestimate the effect of some compounds, while the separation using HPLC is not always able to divide endogenous hormones from xenoestrogens correctly.

Since the EDC biomarker might be a useful tool to understand the relationship between exposure, biological effects and health risks associated with EDCs, it was used: (i) to study its relationship with exposure to EDCs, (ii) to investigate its relationship with adverse health effects, and (iii) to compare exposed populations with not exposed populations. The EDC biomarker showed different associations with exposure to different EDCs. This result can be explained considering that different EDCs may induce different estrogenic effects (agonistic or antagonistic effects), and that the EDC biomarker quantifies the cumulative effect induced by the combination of all these effects. Moreover, this result may also be due to different methodologies applied in different studies (extraction/no extraction and separation/no separation). Indeed, when samples were tested without separation the EDC biomarker also quantified the interaction between EDCs and endogenous hormones, while, when samples were tested after separation, this interaction was not measured. Conflicting results were also found analysing exposed populations (i.e. antagonistic effect measured in most of the Inuit populations, estrogenic activity measured in Faroese women), these results could be due to the use of different estrogenic activity assays (i.e. gene reporter assay in studies on Inuits, E-screen assay in the study on Faroese women). Finally, the EDC biomarker highlighted some possible associations between EDC exposure and diseases (i.e. breast cancer in Spanish women, child adverse health effects).

In conclusion, the estrogenic activity on biological samples may have numerous applications and, depending on the methodology used, it can provide different information:

i) It can be applied as a new method to quantify low levels of E2 in biological samples and for this purpose it should be applied extracting the biological sample;

ii) It can be applied to evaluate the estrogenic status providing considerable insight into both physiological and pathological human conditions and for this purpose it should be applied without any treatment.

iii) It can be applied to evaluate the presence of EDCs in biological samples and for this purpose it should be applied using a separation technique, which allows the separation of endogenous estrogens from xenoestrogens.

iv) It can be applied to evaluate the presence of specific categories of EDCs (such as PFAAs) in biological samples and for this purpose the analytical techniques should be chosen according to the chemical characteristics of the EDCs investigated.

v) It can be applied to assess the combined effect of endogenous estrogens and xenoestrogens in biological samples and for this purpose it should be applied without any separation technique.

Therefore, the choice of methodology implies the evaluation of the research aim and the definition of the estrogenic activity as a biomarker of estrogenic status or EDC exposure.

Studies using this kind of biomarker are still limited in number; however, considering its promising applications, future research is needed in this field in order to improve the standardization of the different methods.

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9. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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11. Table captions

Table 1. Studies applying estrogenic activity assay as estrogen quantification and/or biomarker of estrogenic status.

Footnotes:

AroER tri-screen cells = breast cancer MCF-7 variant without endogenous ER expression (C4-12) transfected with these plasmids: pTomo-ERα vector or pTomo-ERβ vector, pCHGP-2, pCMV-G, pCMV-rev, pGL4.26 [luc2/minP/Hygro] $(ERE)_3$.

Arxula adeninivorans = strain G1212 transformed with YRC102-hER-DsRed2 plasmid.

ERα CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3x ERE-TATA-Luc and pSG5-neo-hERα (Wong et al. 2007).

HeLa transfected = human uterine cervix carcinoma cells (HeLa) stably transfected with pERE4-Luc_{hygro} and pEGFP-$ER\alpha_{neo}$ or pEGFP-$ER\beta_{neo}$ (HeLa ERα transfected or HeLa ERβ transfected, respectively).

HELN = human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively).

MELN = breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid.

Saccharomyces cerevisiae = strain BJ3505 (MATa, ura3-52, tryp1Δ101, lys2-208) transformed with YEPKB1 and YRPE2 plasmids.

Saccharomyces cerevisiae_1 = triply deleted *pdr5 snq2 yor 1* strain transformed with hERα-ERE-GFP or hERβ-ERE-GFP plasmids (Hasenbrink et al. 2006).

T47D-Kbluc = human breast cancer cells (T-47D) transfected with pGL2.TATA.Inr.luc.neo (Wilson et al. 2004).

Table 2. Studies applying estrogenic activity assay as a biomarker of exposure to EDCs.

Footnotes:

BG1Luc4E$_2$ = human ovarian carcinoma cells (BG1) stably transfected with pGudLuc7.0 (Rogers and Denison 2000).

ERα CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3x ERE-TATA-Luc and pSG5-neo-hERα (Wong et al. 2007).

MCF-7 transfected = breast cancer cells (MCF-7) transfected with ERE-tk109 luc, ERE2-tk109 luc, ERE-tk81 luc.

MVLN = breast cancer cells (MCF-7) stably transfected with pVit-tk-Luc and pAG-60 plasmids (Pons et al. 1990).

Saccharomyces cerevisiae_2 = yeast genome integrated with human ER and transformed with plasmid carrying the reporter gene *lac-Z* (plasmid code not specified) (Routledge and Sumpter 1996).

T47D.Luc = human breast cancer cells (T-47D) stably transfected with pERE*tata*Luc.

Table 3. Characteristics of assays for the assessment of estrogenic activity in human biological samples (Mueller 2004, Kiyama and Wada-Kiyama 2015, Seifert et al. 1999, Soto et al. 2006, Wangmo et al. 2018, Wagner et al. 2017). Abbreviations of mammalian and yeast cells reported in table 1 and table 2 (footnotes).

Table 4. Detection limits of estrogenic activity assays compared to detection limits of direct methods for estrogen quantification. Data are expressed as sensitivity for measuring estrogens/progestagens (direct methods) and as sensitivity for measuring E2 (estrogenic activity assays). Ns = not specified. Quantitation limits of direct methods for estrogen

quantification are reported in table S.1 (Supplementary Material). Abbreviations of mammalian and yeast cells reported in table 1 and table 2 (footnotes).